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Novel pathogenic role of fibrin as revealed by a case study on ligneous gingivitis

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ABSTRACT

Purpose of the research: Ligneous gingivitis is a rare disease characterized by nodular gingival enlargement secondary to fibrin deposits induced by micro-injury in the gingiva, which disorder results from plasminogen (PLG) deficiency. Although none have investigated the association of wound healing factors with ligneous gingivitis. In this study, in addition to a histopathologic examination of ligneous gingivitis in a case of type I PLG deficiency, we further present data showing the effect of wound healing factors in association with fibrin *in vitro* to clarify the pathobiology of ligneous gingivitis in PLG-deficient patients.
Principle results: Immunohistochemical analysis revealed that transforming growth factor (TGF)-β1, connective tissue growth factor/CCN2 (CCN2), and endothelin-1 (ET-1) had accumulated in the extracellular matrix around the epithelial and fibroblastic cells near the fibrin deposition. Consistent with these results, fibrin and TGF-β1 synergistically up-regulated CCN2 and ET-1 gene expression in human dermal fibroblasts.
Major conclusions: Fibrin plays a vicious role in ligneous gingivitis pathobiology by up-regulating CCN2 and ET-1 expression through the TGF-β signaling pathway.

1. Introduction

Plasminogen (PLG) plays an important role in wound healing, vascular fibrolysis, cell migration, angiogenesis, and embryogenesis [1]. Type I PLG deficiency is a substantial deficiency of PLG characterized by a proportional reduction in both its immunoreactive PLG level and functional activity. The most common clinical manifestation is ligneous conjunctivitis, characterized by the development of fibrin-rich, ligneous pseudomembranous lesions. Another manifestation is ligneous gingivitis, a rare poorly defined entity characterized by gingival swelling and periodontal tissue destruction, manifestations of which are due to fibrin deposition and abnormal wound healing [2]. The gingival lesions associated with this entity are progressive, usually resulting in tooth loss [3]. Surgical and medical therapies have met with only limited success [4].

Connective tissue growth factor/CCN2 (CCN2) is a heparin-binding 38 kDa secreted protein that plays multiple roles in angiogenesis, chondrogenesis, osteogenesis, oncogenesis, fibrosis, and tissue repair. It is a member of the CCN family of proteins that are characterized by four discrete protein modules, in which reside growth factor binding domains and functional motifs for integrin recognition, heparin and proteoglycan binding, and dimerization [5]. A primary function of CCN2 is to modulate and coordinate the signaling mediated by cell-surface proteoglycans, extracellular matrix (ECM), and growth factors. Abnormal amplification of CCN2-dependent signals results in failure to properly terminate tissue repair, leading to pathological scarring in certain conditions such as fibrosis [5].

Endothelin-1 (ET-1) is a potent vasoconstrictor peptide originally isolated from medium conditioned by cultured vascular endothelial cells. ET-1 is normally produced by endothelial cells, but it has also been shown to be over-expressed during wound

Abbreviations: PBS, phosphate-buffered saline; PLG, Plasminogen; TGF-β1, transforming growth factor β1; CTGF/CCN2, connective tissue growth factor; ET-1, endothelin-1; ECM, extracellular matrix.

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healing [6]. Of note, ET-1 promotes myofibroblast differentiation and ECM production and positively modulates cyclosporin A – induced gingival overgrowth [7].

Upon periodontal tissue injury, an appropriate balance between the production and degradation of the ECM is critically required during the wound healing process. Transforming growth factor β (TGF-β), which is an inducer of CCN2 in normal gingival fibroblasts, is essential for the regeneration of periodontal tissue [8]. Although a few studies have examined the diagnostic utility and developmental roles of these factors, none has investigated their solid association with the disease. Therefore, in this study, in addition to a histopathologic examination of ligneous gingivitis in a case of type I PLG deficiency, we further present data showing the effect of TGF-β1 on the expression of CCN2 and ET-1 in association with fibrin *in vitro* to clarify the pathobiology of ligneous gingivitis in PLG-deficient patients.

2. Case report

A 9-year-old female patient was referred to our clinic with the chief complaint of gingival swelling. At intraoral examination, we found non-painful and nodular fragile ulceration areas affecting both right and left maxillary and mandibular gingiva at the 1st and 2nd molar of deciduous teeth (Fig. 1A–C). She developed pseudomembranes on the palpebral surfaces (Fig. 1D). There was no family history of similar problems in her parents or in her 4-year-old younger sister. No drug use was mentioned. Laboratory evaluation of the peripheral blood of the patient revealed a PLG antigen of 0.3 μg/ml (reference range 9.1–14.5 μg/ml) and a PLG functional activity level of 10% of the normal. For the diagnosis of the gingivitis, a biopsy was carried out at the right lower gingiva under local anesthesia. Hematoxylin and eosin (HE) staining of the gingival biopsy showed ulcer formation and that accumulated homogeneous eosinophilic fibrinous material had filled almost all of the stroma with a slight inflammatory reaction in progress (Fig. 2A and B). Phosphotungstic acid hematoxylin staining showed that a large amount of fibrin was detected (purple: Fig. 2C). Diagnosis was confirmed as ligneous gingivitis.

The patient has been followed up for two and a half years, while the ligneous gingivitis has disappeared, and the legions were replaced with normal gingiva after accomplishment of the natural exchange of deciduous teeth with the following permanent teeth (Fig. 1E). During the course of disease, conjunctival lesions

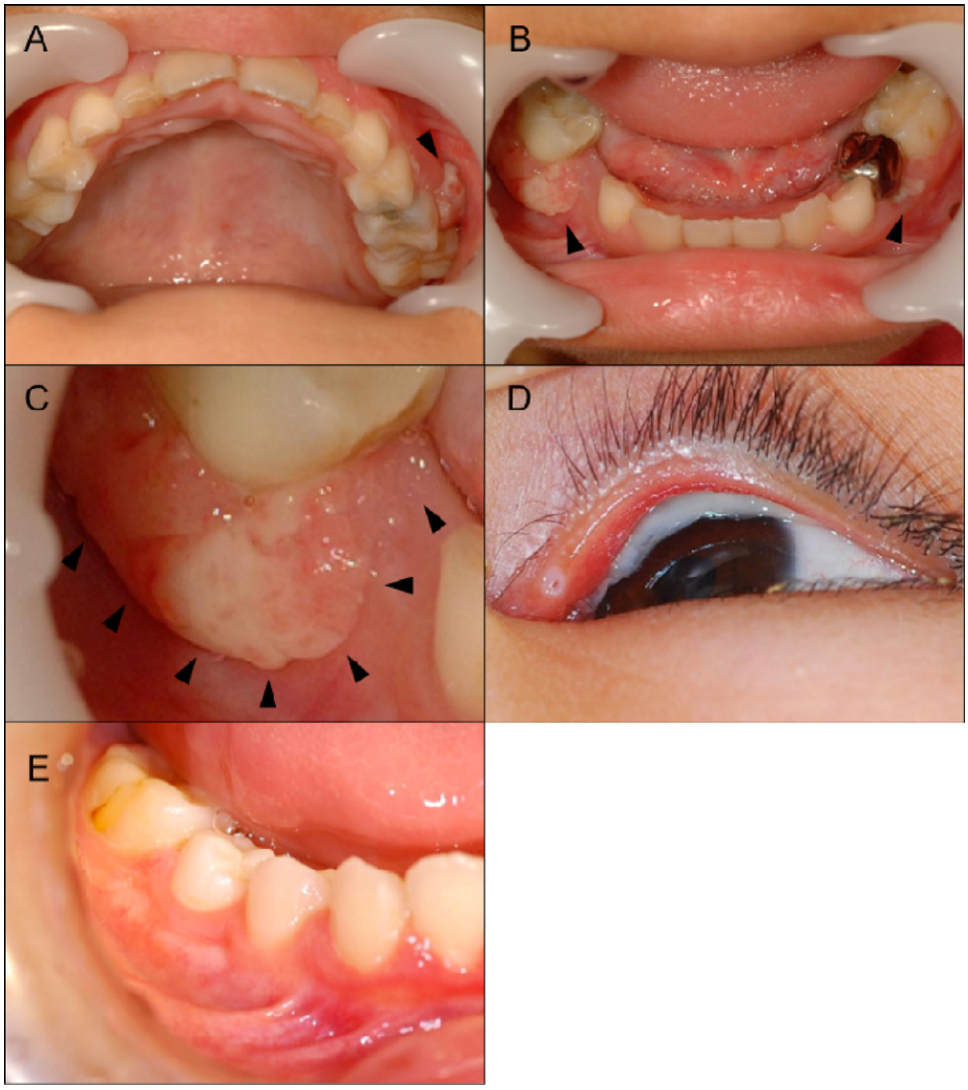

Fig. 1. Clinical findings of the ligneous conjunctivitis and gingivitis in this plasminogen-deficient case. Ligneous gingivitis at the maxilla (A) and mandible (B) (arrowheads). (C) Ligneous gingivitis at the right lower 2nd molar deciduous tooth (arrowheads). (D) A thick pseudomembranous lesion over the palpebral conjunctiva. (E) Intraoral findings of right lower mandible after being followed up for two and a half years. The ligneous gingivitis has disappeared and was replaced with normal gingiva after accomplishment the deciduous teeth removal followed by permanent teeth eruption.

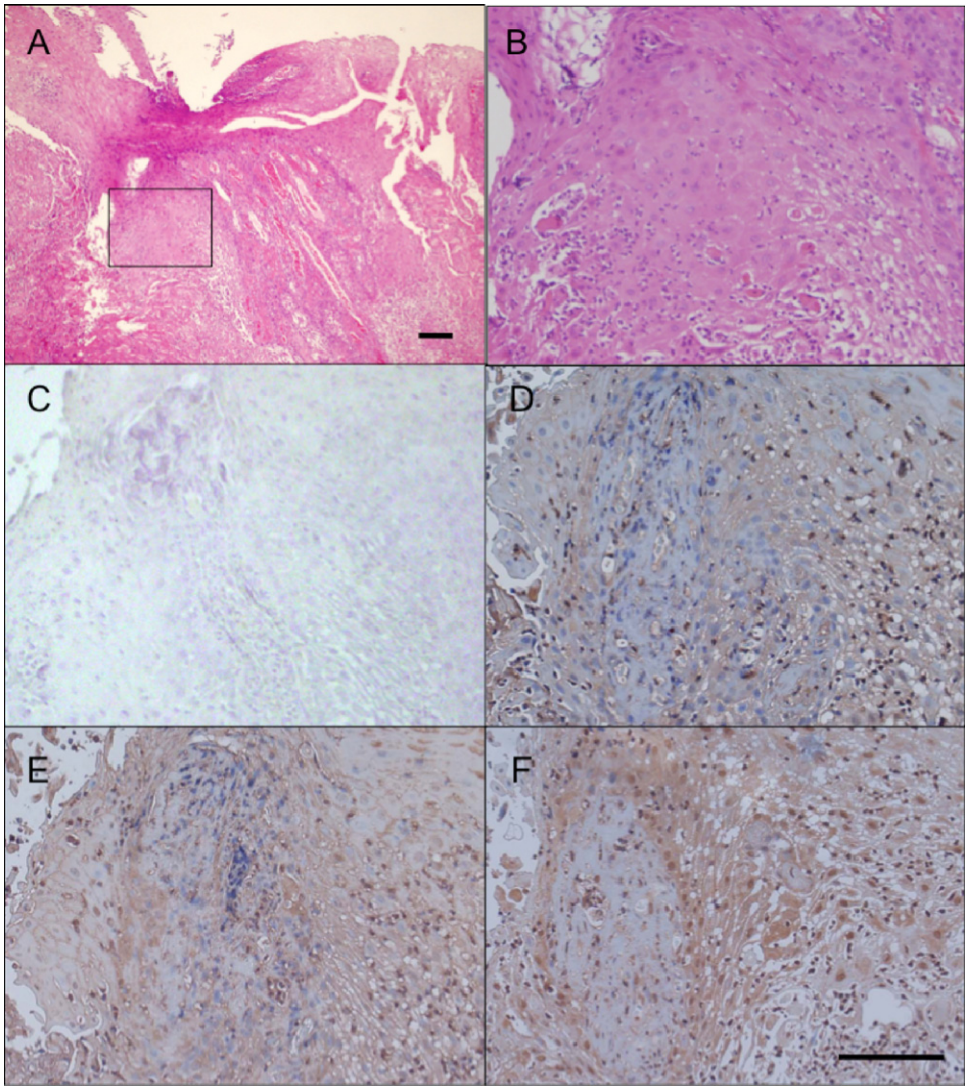

Fig. 2. Histopathological and immunohistochemical examinations of the ligneous gingivitis tissue. (A and B) Hematoxylin and eosin-stained histological section shows ulcer formation and accumulated eosinophilic fibrinous material. (B) Higher magnification of the rectangle area in (A). (C) Phosphotungstic acid hematoxylin staining for fibrin (purple). (D–F) Immunohistochemical staining showing extracellular cell membrane distinctly immunopositive (brown) for transforming growth factor-β1 (D), connective tissue growth factor/CCN2 (E), and endothelin-1 (F). Near fibrin deposition. Scale bar: 200 μm.

have been irrigated by an ophthalmological clinic without further specific therapies. At present, the patient maintains normal visual acuity and good general health.

3. Methods

3.1. Histochemical and immunohistochemical analysis of surgically resected samples

From the surgically resected biopsy samples, HE and phosphotungstic acid hematoxylin staining were performed. A primary anti-TGF-β1 (anti-human antibody, Santa Cruz Biotechnology, Santa Cruz, CA, USA), CCN2 (anti-human antibody, R&D Systems, Minneapolis, MN, USA), and ET-1 (anti-human antibody, Santa Cruz Biotechnology) were used for the immunohistochemical analysis. The immunoreaction was visualized with 3, 3′-diaminobenzidine containing solution as described previously [9].

3.2. Cell culture and immunofluorescence analysis

After having been incubated for 12 h in a 37 °C atmosphere of 5% CO_2/air on culture slides pretreated at 4 °C with 2 μg/ml fibrin from human plasma (Sigma, St Louis, MO, USA) or phosphate-buffered saline (PBS) as a control, primary normal human dermal fibroblasts (LONZA, Walkersville, MD, USA) were washed with PBS and then sequentially fixed for 20 min with cold acetone and permeabilized in 0.1% NP-40 in PBS. After having been washed 3 times with PBS, the fixed cells were incubated at 4 °C overnight with rhodamine-phalloidin and 4,6-diamidino-2-phenylindoledihydrochoride in 3% bovine serum albumin (BSA; Sigma)-PBS and viewed under a fluorescence microscope (IX81, Olympus, Tokyo, Japan).

3.3. RNA extraction and real-time reverse transcriptase-mediated polymerase chain reaction

Total RNA was isolated by using TRIzol reagent (Life Technologies Inc., Carlsbad, CA, USA) according to the manufacturer's instructions. Complementary DNA was generated from 1 μg of total RNA by using the reagents of a First-Strand cDNA Synthesis Kit (Invitrogen, San Diego, CA, USA) in a reaction mixture having a final volume of 20 μl, and was then amplified for 30 cycles by using a pair of oligonucleotide primers: 5′-ACGAGCCCAAGGACCAAA-3′ and 5′-CAGGCAGTTGGCTCTAATCA-3′

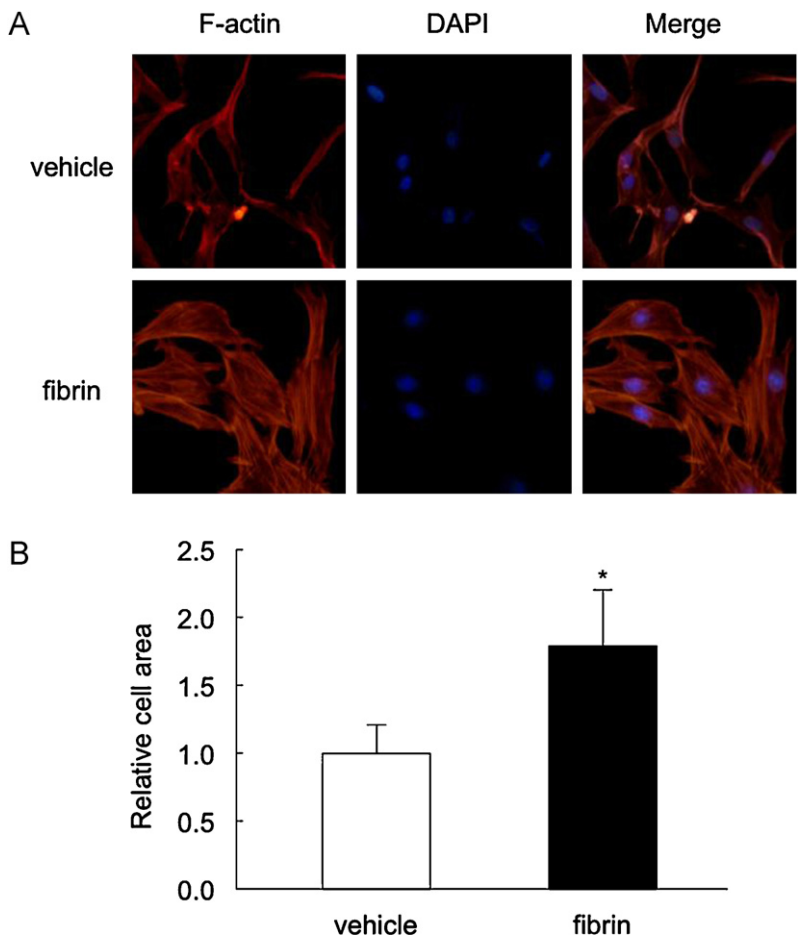

Fig. 3. Structural change in human dermal fibroblasts caused by fibrin attachment. (A) Human dermal fibroblasts were seeded on the surface of tissue culture dishes precoated with bovine serum albumin (2 μg/ml; as a control) or fibrin (2 μg/ml) in Dulbecco's modified Eagle's medium with 1% fetal calf serum. After 24 h, the cells were made permeable and stained with rhodamine phalloidin for the detection of F-actin. Nuclei were visualized with 4,6-diamidino-2-phenylindoledihydrochoride (DAPI). (B) The attached cell area was measured by using NIH Image (National Institutes of Health, Bethesda, MD, USA) and was displayed as a relative ratio against the control (vehicle). $^{*}P < 0.01$.

for CCN2; 5′-TCAGAGGAACACCTAAGACAAACCA-3′ and 5′-CCCGAAGGTCTGTCACCAAT-3′ for ET-1; 5′-TGAACGGGAAGCTCACTGG-3′ and 5′-TCCACCACCCTGTTGCTGTA-3′ for glyceraldehyde-3-phosphate dehydrogenase. Real-time polymerase chain reaction (PCR) was performed with Chroma 4 System (BIO-RAD, Hercules, CA, USA) by using a commercially available master mix containing Taq polymerase and SYBR-Green (Bio-RAD). Each PCR cycle was carried out for 10 s at 95 °C, 5 s at 55 °C, and 10 s at 72 °C.

4. Results

4.1. Expression of wound healing factors in ligneous gingivitis

To analyze the histopathologic characteristics of ligneous gingivitis, immunohistochemistry was performed for representative wound healing factors, TGF-β1, CCN2, and ET-1. Of note, an abundance of TGF-β1 (Fig. 2D), CCN2 (Fig. 2E), and ET-1 (Fig. 2F) accumulated in the ECM around the epithelial and fibroblastic cells near the fibrin deposition.

4.2. Fibrin-attached cells display and altered actin fiber structure

In relation to this case, we examined the biological effect of fibrin on dermal fibroblasts *in vitro*, in order to estimate how the deposited fibrin affected the cytobehavior of these cells. When observed under a fluorescence microscope, fibrin-attached cells displayed an actin fiber structure that was dramatically changed compared with that of the control vehicle-treated group (Fig. 3A). These cells were characterized by a lamellipodial accumulation of F-actin and a flattened cell surface; whereas no such structure was evident in the control cells, which showed the typical fibroblastic morphology. The area of cell attachment in the fibrin-coated group was significantly larger than in the vehicle-treated group ($P < 0.01$, student *t*-test; Fig. 3B).

4.3. Fibrin and TGF-β1 synergistically up-regulate CCN2 and ET-1 gene expression in human dermal fibroblasts

It has been known that CCN2 and ET-1 are induced by TGF-β [10]. Thus, involvement of the activation of TGF-β signaling in the histological findings observed in the ligneous gingivitis patient was suspected. To examine the effects of fibrin on the TGF-β signaling that induces CCN2 and ET-1 gene expression in human dermal fibroblasts, we cultured the cells on fibrin-coated or control dishes for 24 h, treated them with 5 ng/ml of TGF-β, and analyzed the RNA by Real-time reverse transcriptase-PCR (Fig. 4). As a result, the expression of CCN2 gene in the cells was modestly up-regulated by the attachment onto fibrin (day 0, Fig. 4A). Furthermore, induction of the expression of CCN2 and ET-1 by exogenous TGF-β1 was synergistically enhanced by fibrin at 6 and 12 h ($P < 0.01$, Tukey–Kramer, Bonferroni, and Dunnett's test; Fig. 4A and B).

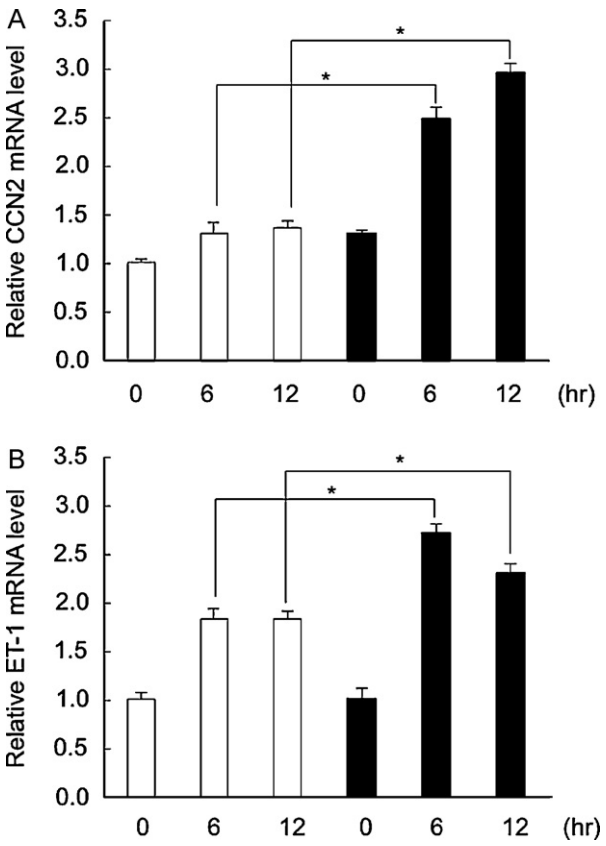

Fig. 4. Induction of connective tissue growth factor/CCN2 and endothelin-1 (ET-1) gene expression in dermal fibroblasts by fibrin attachment and transforming growth factor (TGF)-β. Human dermal fibroblasts were cultured for 24 h in plastic dishes precoated with vehicle (open column) or fibrin (2 μg/ml), solid column in Dulbecco's modified Eagle's medium with 1% fetal calf serum. Thereafter, TGF-β1 (5 ng/ml) was added, and the incubation was continued for an additional 6 or 12 h. CCN2 and ET-1 mRNA levels were determined by real-time reverse transcriptase-polymerase chain reaction analysis. Glyceraldehyde-3-phosphate dehydrogenase was employed as an internal control. $^{*}P < 0.01$.

5. Discussion

Ligneous gingivitis is seen in approximately one-third of patients with homozygous PLG deficiency [2]. The most characteristic finding of ligneous gingivitis is the excessive fragile membranous nodular gingival overgrowths that cause rapid periodontal destruction and consequent tooth loss despite various treatment attempts [3]. This condition may be triggered by minor trauma and is thought to involve fibrin deposition, which plays an important role in hemostasis and wound healing. In this case, the ligneous gingivitis disappeared after the natural removal of deciduous teeth and the following permanent teeth eruption. Thus, careful follow-up to control minor injuries may be the only and critical way to warrant better prognosis of this disorder. Tissue repair is a complex process involving several overlapping stages that include inflammation, cell proliferation, cell migration, angiogenesis, formation of granulation tissue, and production and remodeling of the ECM. Mechanical injury of the skin or mucosa is followed by exudation of plasma and platelet-derived proteins, cleavage of fibrinogen, and fibrin formation. Thereafter, remodeling occurs, and the fibrin matrix is replaced by granulation tissue, and finally, granulation tissue is reconstructed into a secondary matrix. Local extracellular fibrinolysis by plasmin is required for the initial removal of the fibrin-rich matrix as well as for the remodeling of the granulation tissue for the completion of wound healing. Under physiological conditions, the synthesis and degradation of the ECM are adequately balanced in human dermal tissue [11]. However, this balance may be disrupted in ligneous gingivitis patients, which imbalance leads to abnormalities in ECM metabolism, typically characterized by fibrin accumulation. Thus, the alteration in ECM composition may be ascribed to the abnormal wound healing process at least in part.

When fibroblasts are stimulated in an injured tissue environment, cell migration, DNA synthesis, and protein production are enhanced, causing an overall increase in cell size. In this study, human dermal fibroblasts displayed a structural change in their actin fibers when attached onto fibrin *in vitro*. The observed actin reorganization appeared to lead to the formation of lamellipodia for migration. For efficient migration, predeposition of ECM as a scaffold is required; hence this structural change is likely to be associated also with an increased capacity of the cells to synthesize ECM. This hypothesis is supported by the observed accumulation of TGF-β that promotes ECM synthesis.

The development of ligneous gingivitis involves complex interactions between fibrin, cells, and cytokines. Here, we report that a large proportion of the fibroblasts in the ligneous gingivitis patient were TGF-β-producing ones. TGF-β1 is a key cytokine for wound healing. It is produced by all the major cell types participating in wound repair, including T-lymphocytes, macrophages, smooth muscle cells, endothelial cells, epithelial cells, and fibroblasts. Fibroblasts are the predominant cell type involved in wound healing; and especially, most of the TGF-β1 in ligneous gingivitis lesions can be supplied by these cells through an auto-induction feedback loop. Integrins change their expression pattern under pathological conditions and contribute to the regulation of fibrogenesis via modulating ambient TGF-β activity [12,13]. The regulation of various signals downstream of integrin-fibrin engagement may modulate TGF-β signaling during wound healing, although the precise mechanisms of synergistic up-regulation of CCN2 and ET-1 by TGF-β1 need to be further investigated. Previous reports show that TGF-β1 and CCN2 or ET-1 coordinately function together to regulate wound healing [6,8]. CCN2 exhibits numerous biological properties of potential importance for the wound healing response, which include stimulation of cell proliferation, cell adhesion, chemotaxis, angiogenesis, and production of ECM components. In fact, CCN2 and ET-1 overproduction also results in enhanced wound closure and collagen deposition, as does TGF-β1. CCN2 is involved in ET-1-induced ECM accumulation, not only in a direct manner, but also through downstream effectors [5]. Of note, CCN2 binds to fibronectin (FN) and enhances the affinity of FN for fibrin [14]. These results indicate that CCN2 appears to contribute to the ECM accumulation in abnormal wound healing in ligneous gingivitis, together with fibrin, TGF-β1, and ET-1.

6. Conclusion

In conclusion, ligneous gingivitis, which is usually seen in patients with ligneous conjunctivitis, is a rare disease characterized by gingival overgrowths and periodontal destruction due to PLG deficiency and subepithelial accumulation of fibrin. Based on the results on the examination of TGF-β1, CCN2, and ET-1 accumulation in this patient with ligneous gingivitis and the analysis *in vitro*, we propose that fibrin plays a vicious role in the disease by up-regulating CCN2 and ET-1 gene expression through TGF-β signaling pathways; although how fibrin causes TGF-β accumulation remains to be investigated.

Conflict of interest

None of the authors have any conflict of interest.

Acknowledgment

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